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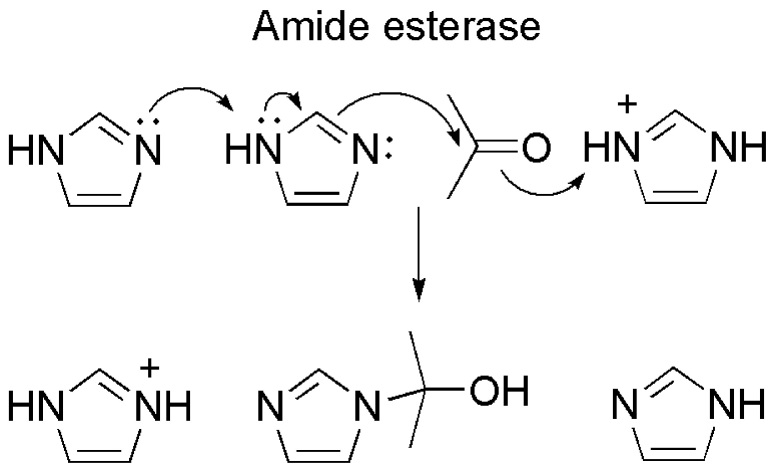


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# Covalent Intermediates and Enzyme Proficiency

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How enzymes catalyze reactions has been of great interest for over a hundred years. Both ground state and transition state contributions to the rate of enzyme-catalyzed reactions have been considered. In 1894, Emil Fischer proposed that the substrate fits into the enzyme like a key fits into a lock.[1] In the language of the present age, the Fischer proposal would be: substrate conformers resembling the transition state fit the enzyme active site like a key fits a lock but with a little wiggle. Such conformers have been called near attack conformers (NACs) and are readily observed by molecular dynamics.[2] The free energy ($\Delta G^{\ddagger}$) for conversion of the Michaelis complex (E·S) to the enzyme transition state complex (E·TS) is the sum of the standard free energy for NAC formation plus the free energy of activation for E·NAC → E·TS (eq 1).

$$\Delta G^{\ddagger} = \Delta G^{\circ}_{\mathrm{NAC}} + \Delta G^{*}_{\mathrm{TS}} \qquad (1)$$

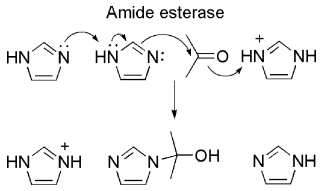
Linus Pauling (1946, 1948) proposed[3] that an enzyme has the specificity of an antibody in recognizing the shape of and charges on the TS, and the resulting interactions between the enzyme and the TS stabilize the TS. In addition to stabilization by recognition, the TS may be altered to become a more stable TS by general acid, general base, and nucleophilic catalysts, etc. Over the years, all methods proposed to decrease $\Delta G^{\ddagger}$ have been collected together under the single term "transition state stabilization" such that the term is no longer very specific.

Spector (1982), in his book "Covalent Catalysis by Enzymes", proposed[4] that "all enzyme reactions proceed through at least one intermediate in which the enzyme is covalently joined to its substrate or a fragment thereof". Much of what Spector presented was sound chemistry, however, much was not. For example, hydride equivalent transfer from NAD(P)H does not involve $2e^-$ transfer followed by covalent bond formation and then $H^+$ transfer. Certainly all enzymatic reactions do not involve enzyme−covalent intermediates.

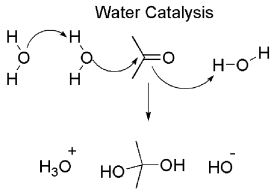
Zhang and Houk[5,6] have proposed that ground state conformations and TS stabilization cannot explain very large enzyme efficiencies. They point out that formation of covalent enzyme−substrate intermediates, enzyme−substrate−cofactor intermediates, etc., are associated with the greatest enzyme efficiencies. (Their definition of covalent intermediate is at times very loose,[6] including some hydrogen bonds and metal ligation.) Thus, the formation of covalent enzyme−substrate intermediates is offered as an explanation for large enzyme efficiencies and is proposed to be the most important aspect of enzymatic catalysis.

There are two principal ways to describe the catalytic ability of an enzyme. One is to compare the rate constant of one enzyme to that of another. The other is to compare the efficiency of one enzyme to that of another. By convention, the efficiency of an enzyme is defined as the rate constant ($k_{\mathrm{cat}}/K_{\mathrm{m}}$) for the enzymatic reaction divided by the rate constant ($k_{\mathrm{o}}$) for conversion of substrate to product in water at pH 7.0. The standard free energy for NAC formation ($\Delta G^{\circ}_{\mathrm{NAC}}$) is not a major feature in determining the free energy of activation for an enzymatic reaction because the term is usually less than 1−2 kcal/mol. However, when comparing rate constants for enzymatic and water reactions, one must consider this feature. To determine the relative importance of NAC formation, one compares both $\Delta G^{\circ}_{\mathrm{NAC}}$ and $\Delta G^{*}_{\mathrm{TS}}$ of eq 1 for both the enzymatic reaction and the reaction in water at pH 7.0. In doing so, one finds that the standard free energy for NAC formation is always more favorable in the enzyme than in water. Thus, to some extent, the enzyme efficiency is dependent upon the ability of the enzyme to form NACs relative to the ability for NACs to be formed in water. Recent results[7] demonstrate that the advantage of chorismate mutase enzymes in forming NACs provides 90% (∼8 out of 9 kcal/mol) of the free energy advantage ($\Delta\Delta G^{\ddagger}$) of the enzymatic reaction over that in water.

***Scheme 1***




***Scheme 2***




If the proposal by Zhang and Houk has meaning, the mechanisms of the most reactive enzymes should involve covalent intermediates. In a collection of 24 enzymes,[8] values of $k_{\mathrm{cat}}/K_{\mathrm{M}}$ are between $10^5$ and $10^9$ $M^{-1}$ $s^{-1}$. Among the most reactive enzymes are those that do not involve the formation of covalent intermediates. A few examples are fumarase ($k_{\mathrm{cat}}/K_{\mathrm{M}} > 10^9$ $M^{-1}$ $s^{-1}$),[9] orotidine 5-phosphate decarboxylase ($\sim 10^8$ $M^{-1}$ $s^{-1}$),[10−12] and staphylococci nuclease ($k_{\mathrm{cat}}/K_{\mathrm{M}} > 10^7$ $M^{-1}$ $s^{-1}$).[13] From these data, it is apparent that there are enzymes that catalyze very rapid reactions that do not form covalent intermediates.

Because enzyme efficiency is, by convention, the rate constant for the enzymatic reaction divided by the rate constant in water, efficiency becomes greater when $k_{\mathrm{cat}}$ becomes greater or when $k_{\mathrm{o}}$ becomes smaller. The rate constants range over only about $10^4$ for a group of enzymes whose efficiency half-life ranges from 1 min to 1 billion years. The much greater difference in enzyme efficiencies must relate not to the enzymatic reaction but to the reaction in water.[14] Water is a very poor nucleophile, and at pH 7.0, the concentrations of acid ($H_3O^+$) and base ($HO^-$) are $10^{-7}$ M. The more complex the enzymatic reaction, the more roles water must play in the water reaction. As an example, let us compare the hypothetical "amidesterase" of the trihistidine family and its water counterpart (Schemes 1 and 2).

In Scheme 1, nucleophilic addition of an imidazole (HIm) to the carbonyl group is general base catalyzed by another HIm and



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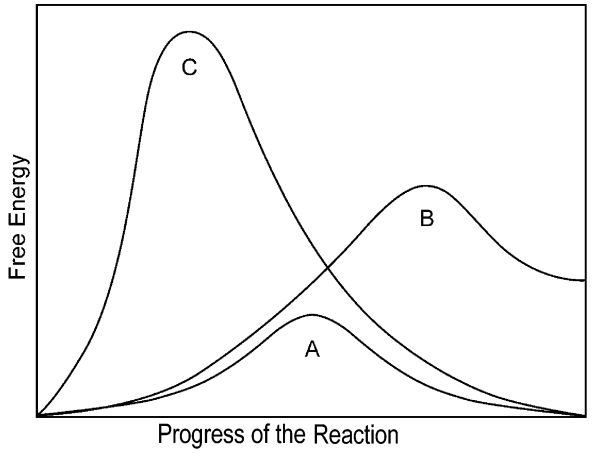


***Figure 1.*** Cartoon reaction coordinates. (A) Catalysis of imidazole addition to carbonyl by imidazole general base and imidazolium general acid. Starting imidazole and imidazolium species are regenerated as product. (B) Catalysis of water addition to carbonyl by water molecules. The imidazole and water reactive conformations are equally possible, but the immediate product of the water reaction includes $HO^-$ and $H_3O^+$ species. (C) Nucleophilic addition of water to carbonyl catalyzed by hydroxide ion and hydronium ion. The probability of forming the starting $HO^-\cdots S\cdots HOH^+$ complex is extraordinarily small.

general acid catalyzed by an imidazolium ion ($HImH^+$). In this enzyme, the p$K_a$ of the $HImH^+$ is 6.7; therefore, both HIm and $HImH^+$ are readily available at pH 7.0. Replacing both HIm and $HImH^+$ with water molecules (Scheme 2) provides a reaction in which water molecules play the role of both the acid and base catalysts as well as the nucleophile. On examination of the two reactions, one would surmise that NAC formation in the enzyme and in water would be comparable. However, the water reaction suffers in that water is not as good a base as imidazole (HIm) and not as strong an acid as the imidazolium ion ($HImH^+$). If the Brønsted $\alpha$ and $\beta$ constants are between 0.5 and 0.7, then $k_{cat}$ will exceed $k_o$ by $10^{10}$ to $10^{17}$ (the enzyme efficiency). An alternate scheme for the water reaction would have $H_3O^+$ and $HO^-$ as acid and base catalysts. Since these two species would both be present at $\sim 10^{-7}$ M at pH 7.0, a trimolecular complex of $HO^-$ and $H_3O^+$ and substrate would, for all practical purposes, never form. Figure 1 represents a cartoon of the free energies versus the reaction coordinates we have considered. In the case of the serine esterases, water molecules would replace Asp-$CO_2^-$, Hist-Im, and Ser-OH; thus, the enzymatic reaction would be compared to the hydrolysis of a peptide bond in water at pH 7.0 ($k_o \sim 10^{-10}$ $s^{-1}$).[15]

The enzyme efficiency is defined as the ratio of the rate constant for the enzymatic reaction and rate constant for the reaction in water at pH 7.0. Complex enzymatic reactions may have efficiencies much greater than simpler enzymatic reactions because water is a poor nucleophile with little acid or base property. The more complex the enzymatic reaction, the more roles water must play badly. In addition, for those reactions where the substrate must be guided to a reactive conformation (NAC), the enzymatic reaction has the greater advantage. Comparison of enzyme efficiencies is not to be confused with comparison of enzymatic rate constants. The numerical rate constants for enzymatic reactions do not depend on whether covalent intermediates are involved. An enzyme is much like a chemist; for both, acyl transfer reactions involve covalent tetrahedral intermediates, and phosphodiesterase bond scissions require pentacoordinate intermediates, which are very transition-state-like in structure. These reactions are not known, in the laboratory or at the active site of the enzyme, to be exceptionally rapid. They were selected in the development of the enzymes not to provide large rate constants but as the only way to get to the product. In short, covalency is a requirement of mechanism, and we must remember that each step in a mechanism has a TS whose formation may require electrostatic assistance and/or acid/base catalysis and is subject to the stabilizations referred to by Pauling. The huge collection of data by Zhang and Houk will be of treasure in future research. However, the covalent proposal does not provide additional insight to enzymatic catalysis. In short, Zhang and Houk[5] have not taken into account that high enzyme efficiency is determined by the value for the water reaction $k_o$ rather than by the enzymatic rate constant $k_{cat}/K_M$.

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